

Original Article

Chromogranin A in the Pancreatic Islet: Cellular and Subcellular Distribution¹

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Chromogranin A (CGA) is the major soluble protein within secretory vesicles of chromaffin cells. A polyclonal antiserum was raised against bovine CGA and characterized in two-dimensional immunoblots. Cellular and subcellular distribution of CGA in bovine pancreatic islet was investigated by immunocytochemistry. At the light microscopic level, CGA-like immunoreactivity was found in the same cells that react with antibodies against insulin, glucagon, and somatostatin. A minority of cells containing pancreatic polypeptide also showed faint immunostaining. At the ultrastructural level (protein A-gold technique), CGA-like

immunoreactivity was confined exclusively to the secretory vesicles. Whereas the hormones were localized mainly in the central part of the secretory vesicles, CGA was present predominantly in the periphery. These findings indicate that a CGA-like protein is a regular constituent of the matrix of secretory vesicles in pancreatic endocrine cells. (*J Histochem Cytochem* 34:1673, 1986)

KEY WORDS: Chromogranin A; Endocrine pancreas; Bovine; Immunostaining; Cellular and subcellular co-localization with hormones.

Introduction

Chromogranin A (CGA) is an acidic protein which is stored and released with catecholamines from chromaffin cells. It is the main protein contained in the chromaffin vesicle and is part of the Ca²⁺ storage complex in the matrix of chromaffin vesicles (Reiffen and Gratzl, 1986a, 1986b; Bulenda and Gratzl, 1985). Its function therefore resembles that of calsequestrin in the sarcoplasmic reticulum.

Within the past few years, CGA has also been detected by various techniques in many other endocrine cells and tissues including the pituitary, the endocrine pancreas, the intestine, the thyroid, and the hypothalamus (O'Connor, 1983). Furthermore, secretory protein I, a protein released from the parathyroid gland, turned out to be chemically closely related to, if not identical with CGA (Cohn et al., 1982, 1984). CGA was immunohistochemically located in the thyroid parafollicular C-cells, parathyroid chief cells, pancreatic endocrine cells, entero-endocrine cells, and anterior pituitary cells (Nolan et al., 1985; Cohn et al., 1984; Wilson and Lloyd, 1984; O'Connor et al., 1983).

Within the endocrine pancreas, the cellular location of chro-

mogranin A remained unclear. O'Connor et al. (1983) found CGA-like immunoreactivity in insulin-producing B-cells; Wilson and Lloyd (1984) and Varndell et al. (1985) described only weak staining in B-cells but intense immunostaining in the glucagon-producing A-cells. Cohn et al. (1984) attributed CGA immunoreactivity to somatostatin- and pancreatic polypeptide-producing cells of the islets. To clarify this issue, we examined immunohistochemically the distribution of this protein in the bovine endocrine pancreas and compared it with that of the established pancreatic hormones insulin, glucagon, somatostatin, and pancreatic polypeptide. Using the protein A-gold technique, we were also able to determine the location of the antigen on the subcellular level.

Materials and Methods

Preparation and Characterization of Antichromogranin A. Chromogranin A was purified from the soluble content of isolated bovine chromaffin vesicles. Dopamine- β -hydroxylase was removed by passage through a Concanavalin A-Sepharose 4B column (Aunis and Miras-Portugal, 1976). CGA was separated from the bulk of chromogranins by one-dimensional SDS-polyacrylamide gel electrophoresis and eluted electrophoretically from gels. Upon re-electrophoresis, we observed the 70 kD CGA band plus two faint bands (47 and 58 kD) corresponding to the CGA breakdown products. CGA (500 μ g) was emulsified with complete Freund's adjuvant and administered intradermally at 25–30 injection sites on the back of the rabbit *Europium*. Three weeks later, 550 μ g of CGA was again injected intradermally, but on the contralateral side of the back. Ten days later the animal was bled.

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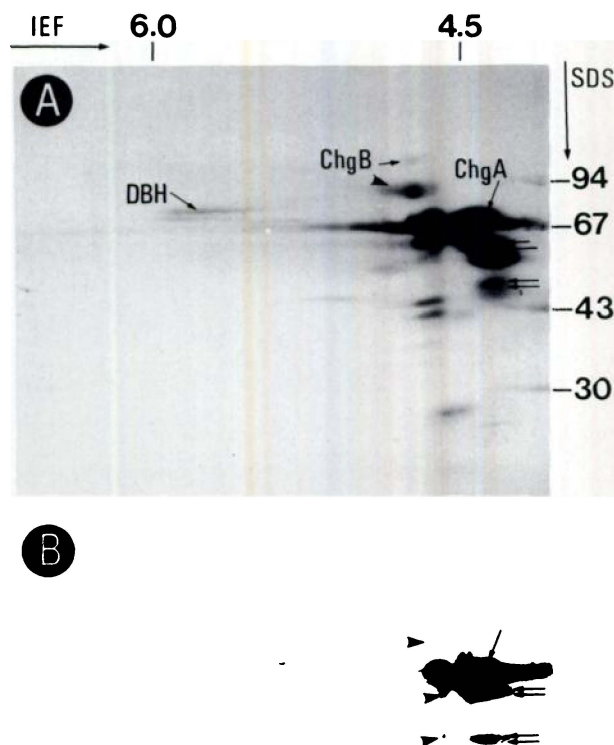


Figure 1. Biochemical characterization of the anti-CGA antiserum. (A) Two-dimensional gel electrophoregram of chromaffin granule soluble proteins. IEF, isoelectrofocusing in the first dimension; SDS, sodium dodecylsulphate-slab gel electrophoresis of focused proteins on 8–15% polyacrylamide gradient gel in the second dimension. ChgA, chromogranin A (70 kD Mr); ChgB, chromogranin B (100 kD Mr); DBH, dopamine β -hydroxylase (72 kD Mr). Chromogranin B is defined as described by Fischer-Colbrie and Frischenschlager, 1985. (B) Immunoreplica labeled with anti-chromogranin A antiserum. Double arrows indicate immunoreactive CGA breakdown products (47 and 58 kD Mr), while arrowheads show faint immunoreactive chromogranin B breakdown products. *Europium* antiserum was used at 1:20,000 dilution and 500 μ g soluble granule proteins were deposited on the rod gel. At a 1:40,000 dilution, no immunostaining of chromogranin B degradation products was detectable.

The antiserum was tested by immunoblotting. Soluble proteins (500 μ g) were separated by two-dimensional gel electrophoresis and transferred to nitrocellulose sheets. Immunoreactive proteins were detected using *Europium* antiserum at a dilution of 1:1000 and horseradish peroxidase-coupled goat anti-rabbit immunoglobulins (Nordic Immunological Laboratories, Tilburg, Netherlands; dilution 1:1000). Color reaction was developed with chloro-2-naphthol and H_2O_2 as described by Bader and Aunis (1983).

Immunohistochemical Techniques. Small specimens from bovine pancreas were quenched in liquid Freon 22 at -150°C , freeze-dried for 72 hr, and then fixed by vapor-phase paraformaldehyde. Serial semi-thin sections 0.5 μ m wide were cut from tissue blocks embedded in epoxy resin. After complete removal of the resin by Na methoxide (Mayor et al., 1961), the sections were sequentially immunostained for the established pancreatic hormones insulin, glucagon, somatostatin, and bovine pancreatic polypeptide (BPP) and for CGA using the peroxidase-antiperoxidase (PAP) method as modified for semi-thin sections (Grube, 1980). The an-

tisera were used at dilutions of 1:1000 (anti-insulin), 1:2000 (anti-glucagon), 1:4000 (anti-somatostatin and anti-bovine PP), and 1:2000–1:64,000 (anti-CGA). Specimens from bovine adrenal medulla prepared in the same way served as positive controls for immunostaining with the CGA antiserum. Specificity controls included all tests to exclude method and antibody nonspecificities (see Grube, 1980).

For immunoelectron microscopy, small pieces of bovine pancreas were fixed with Acrolein (5% Acrolein, 0.1 M phosphate buffer, 2% sucrose) for 1 hr (King et al., 1983) and embedded in epoxy resin. Adjacent ultra-thin sections were mounted on 200-mesh uncoated nickel grids and attached to the grids by heat (90°C for 30 min). The resin was partially removed by immersion for 6 sec in Na methoxide (Mayor et al., 1961) diluted 1:5 (vol/vol) in a mixture of equal parts of methanol and benzene. After short rinses in methanol/benzene, acetone, and water, the grids were placed for 15 min in 1% ovalbumin (Sigma; St. Louis, MO) diluted in phosphate-buffered saline (PBS). They were then incubated overnight in the primary antibody which was diluted in 0.5% ovalbumin in PBS. The antisera were diluted 1:8000 (anti-insulin), 1:50,000 (anti-glucagon), and 1:10,000 (anti-CGA). After rinsing in PBS, they were incubated for 1 hr in protein A-gold solution and then rinsed again in PBS and in water. The contrast in the sections was increased by applying uranyl-acetate and lead citrate. The sections were examined with a Zeiss EM 10 and a Phillips EM 301 at 80 kV acceleration voltage. For subcellular localization of CGA in somatostatin-producing D-cells, the consecutive semi-thin/ultra-thin section technique (Lange, 1967) was applied and adjacent sections were immunostained for somatostatin or CGA. Distribution of the gold particles on the vesicles was determined from prints of serial sections. For each considered antigen, the gold particles on 50 vesicles were counted. Two zones of the vesicle matrix were analyzed: the periphery and the center. In insulin-containing vesicles, the periphery was defined as the distinctly delineated clear halo and the outer layer of the dense core. In glucagon-containing vesicles, the periphery was established to be the outer 20% of the vesicles' diameter.

The protein A-gold complex was prepared according to the method of Roth (1978 and personal communication). Colloidal gold was made by reduction of 100 ml 0.01% aqueous solution of tetrachloroauric acid (Merck; Darmstadt, FRG) with 4 ml 1% Na acetate (Frens, 1973). The colloidal gold was stabilized with 0.6 μ g protein A per ml gold solution.

The insulin antiserum, the glucagon antiserum, and the somatostatin antiserum used for immunoelectron microscopy were kindly supplied by Dr. Jens Holst, Copenhagen. The somatostatin antiserum used for immunocytochemistry on the light microscopic level was obtained from Dr. Etzrodt, Ulm, and the BPP antiserum from Dr. R.E. Chance, Indianapolis.

Results

The anti-CGA antibody was characterized in an immunoblot after two-dimensional separation of chromaffin vesicle matrix proteins. As seen in Figure 1, a dense precipitate was observed at the spot corresponding to CGA. We also noted the staining of CGA breakdown products (arrows in Figure 1b; 59 kD, 55 kD, and 45 kD). A very faint staining of a chromogranin B product with a molecular weight of 80 kD was observed, but not, however, with chromogranin B itself (arrowhead, Figure 1a).

The antiserum was also tested in tissue sections of bovine adrenals at the light microscopic level (PAP technique). Here, even dilutions of the CGA antiserum of up to 1:64,000 resulted in positive staining of the adrenal medulla cells (Figures 2a and 2b). At the ultrastructural level (protein A-gold technique), the secretory vesicles within all the chromaffin cells were densely labeled (Figure 2c), whereas other structures had negligible amounts of gold

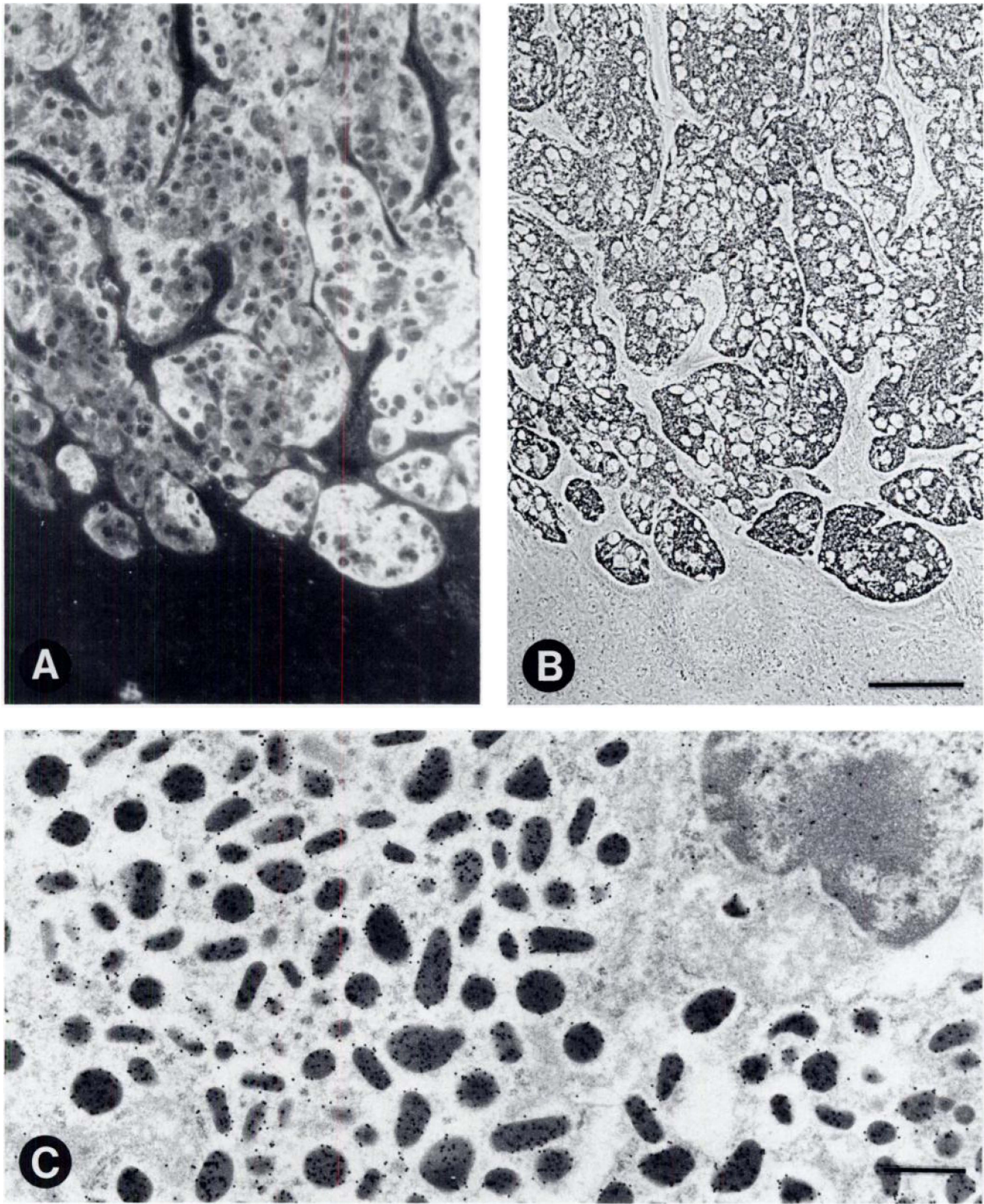


Figure 2. Immunocytochemical characterization of the anti-CGA antiserum. Two adjacent semi-thin ($0.5\ \mu\text{m}$) sections of bovine adrenal medulla. (A) Formaldehyde-induced fluorescence of the medulla cells; (B) All fluorescing cells show (irrespective of their actual amine content) intense immunostaining for CGA. Phase-contrast microscopy. Bar = $50\ \mu\text{m}$. (C) All chromaffin vesicles of the adrenal medulla display immunostaining for CGA (protein A-gold technique), whereas other structures have negligible amounts of gold particles. Bar = $0.5\ \mu\text{m}$.

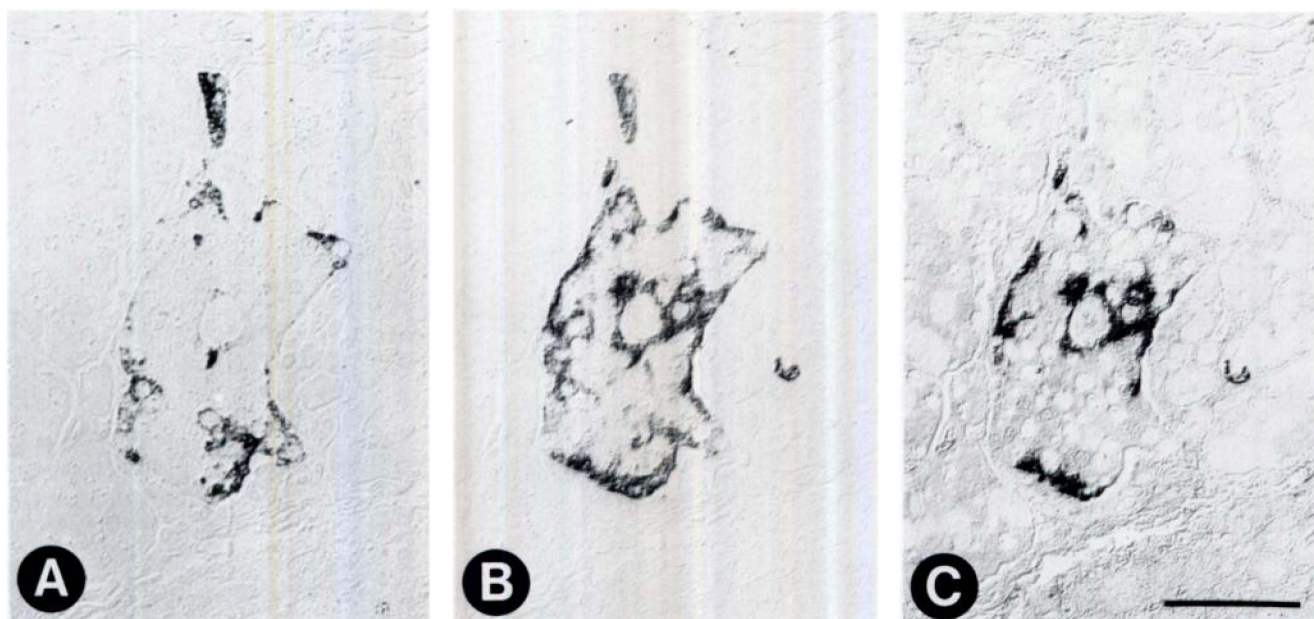


Figure 3. Three adjacent semi-thin (0.5 μ m) sections of an islet of the bovine pancreas. The sections were immunostained for insulin (A), CGA (B), and glucagon (C). All insulin and glucagon cells are immunoreactive for CGA. Interference contrast microscopy. Bar = 50 μ m.

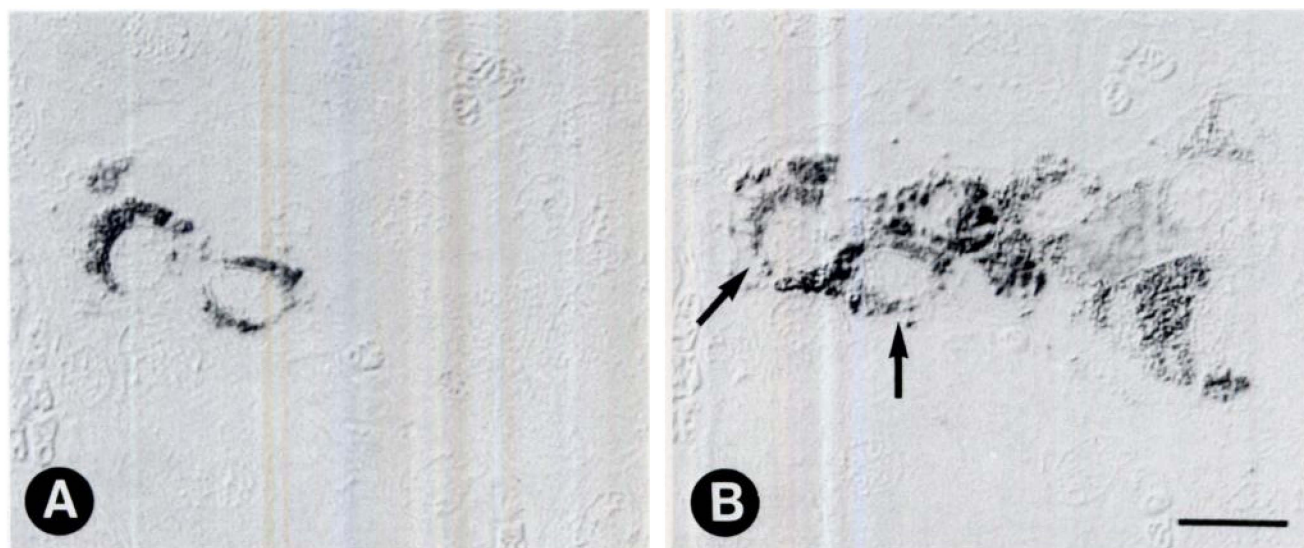


Figure 4. Two adjacent semi-thin (0.5 μ m) sections of calf pancreas. Somatostatin-immunoreactive cells (A) show low-intensity CGA immunoreactivity (B). In B the somatostatin cells are marked by arrows. Interference contrast microscopy. Bar = 10 μ m.

particles. In the secretory vesicles, reaction product was found throughout the matrix.

Within the pancreas, the CGA antiserum could also be used in dilutions of up to 1:64,000 for positive staining. CGA-like immunoreactivities were confined exclusively to the endocrine cells (islets of Langerhans and single endocrine cells dispersed in the exocrine parenchyma). There was no reaction product in the exocrine part of the gland. This observation is consistent with earlier observations by other groups (O'Connor et al., 1983; Wilson and Lloyd, 1984; Cohn et al., 1984). Examination of sequentially im-

muno-stained serial semi-thin sections revealed that all insulin- and glucagon-producing cells of bovine endocrine pancreas react heavily with the CGA antiserum (Figure 3). CGA-like immunoreactivity was also observed in all somatostatin (D-) cells. However, staining intensity was lower in these D-cells than in the insulin (B-) and glucagon (A-) cells (Figure 4). The majority of cells containing bovine pancreatic polypeptide (PP-cells) were unreactive to the CGA antibody. Only a minority of PP-cells displayed immunoreactivity for CGA, and that was mostly of low intensity or only on certain secretory vesicles (see arrow in Figure 5).

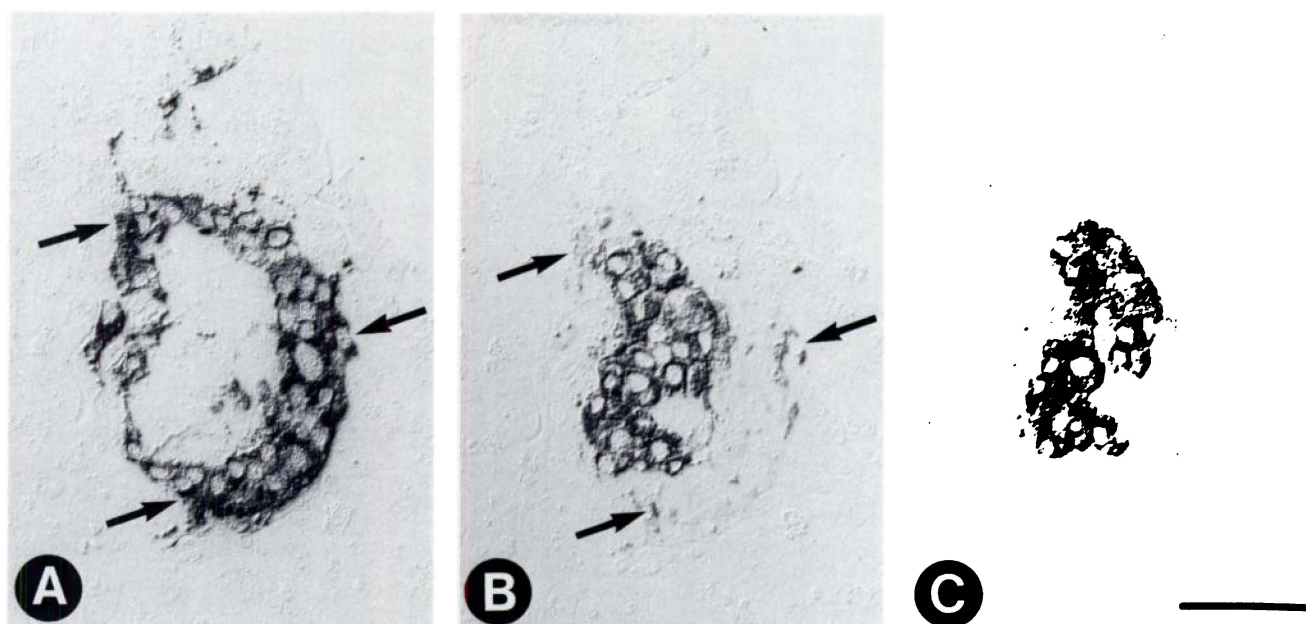


Figure 5. Three serial semi-thin (0.5 μm) sections of an islet of calf pancreas. The sections were immunostained for pancreatic polypeptide (A), CGA (B), and insulin (C). This islet is composed exclusively of insulin and pancreatic polypeptide cells. Only a few PP-cells or some secretion granules of PP-cells are immunoreactive for CGA (arrows in A and B). The centrally located CGA-immunoreactive cells of this islet are insulin cells (C). Interference contrast microscopy. Bar = 50 μm .

At the ultrastructural level, CGA-like immunoreactivity was located exclusively in secretory vesicles of the pancreatic endocrine cells. No staining was observed in the cytoplasm or other subcellular structures. To examine the possible co-localization of CGA and the pancreatic hormones, adjacent ultra-thin sections immunostained for insulin or glucagon and for CGA were compared. All the glucagon- and insulin-containing vesicles proved to contain CGA also (Figures 6 and 7). The weaker immunoreaction observed in the somatostatin-containing vesicles corresponds to the same observation at the light microscopic level (Figure 8).

The topography of the gold particles within the vesicles depended on the type of endocrine cell tested: $71.3 \pm 2.3\%$ (mean \pm SEM) of the insulin immunoreactivity was located in the electron-dense core of the vesicles. In contrast, $65.5 \pm 3.2\%$ (mean \pm SEM) of the CGA-like immunoreactivity was observed in the clear halo and outer layer of the hormone-containing core (Figure 9). Likewise, glucagon immunoreactivity was found to be concentrated in the center of the vesicles ($66.7 \pm 4.1\%$; mean \pm SEM), whereas $66.4 \pm 2.8\%$ (mean \pm SEM) of the CGA immunoreactivity was present in the peripheral zone of these vesicles (Figure 10). In the paired *t*-test, the different results obtained for CGA in the core versus either insulin or glucagon were highly significant ($p < 0.001$).

Discussion

Within the past few years it has become evident that CGA originally detected in the adrenal medulla (Smith and Kirshner, 1967; Smith and Winkler, 1967; Banks and Helle, 1965) is also a typical component of other endocrine tissues, such as the endocrine pancreas. However, as pointed out above, the cellular distribution of CGA in pancreatic endocrine cells remained unclear. Moreover, its

subcellular location in the hormone-producing cells of the endocrine pancreas was unknown. Our results, which have been proven to be specific during all specificity controls (see Grube, 1980), indicate the CGA is present in all insulin-, glucagon-, and somatostatin-producing cells, but only in a minority of all PP-immunoreactive cells.

These results differ from that obtained by previous investigators: O'Connor et al. (1983) ascribed CGA immunoreactivity mainly to B-cells, whereas Wilson and Lloyd (1984) found only weak immunostaining in B-cells but intense immunoreactivity in the glucagon-producing A-cells. Cohn et al. (1984) located secretory protein I/CGA immunoreactivity in somatostatin- and pancreatic polypeptide-producing cells.

These discrepancies may arise from interspecies variations in immunoreactivity for the antiserum used. We made corresponding observations when we performed comparative light microscopic immunohistochemical studies in the pancreas of other mammals (including dog, rabbit, guinea pig, rat, and human) with our CGA antiserum. During these studies (unpublished observations), we found that the reaction of our antibovine CGA antiserum with the different types of pancreatic endocrine cells depends on the species. It remains as yet unclear whether the lack of immunoreactivity in certain endocrine cells of other species and the variable immunoreactivity of bovine pancreatic PP-cells towards bovine CGA antibodies are caused by the presence of a molecular variant of CGA or by a "masking" of CGA in these locations. Another explanation for the inconsistent observations may be that there is different expression of the related protein families chromogranin A, B, and C in different cells or species. An example of chromogranins' species dependence has recently been given by Fischer-Colbrie and Frischenschlager (1985), who showed that

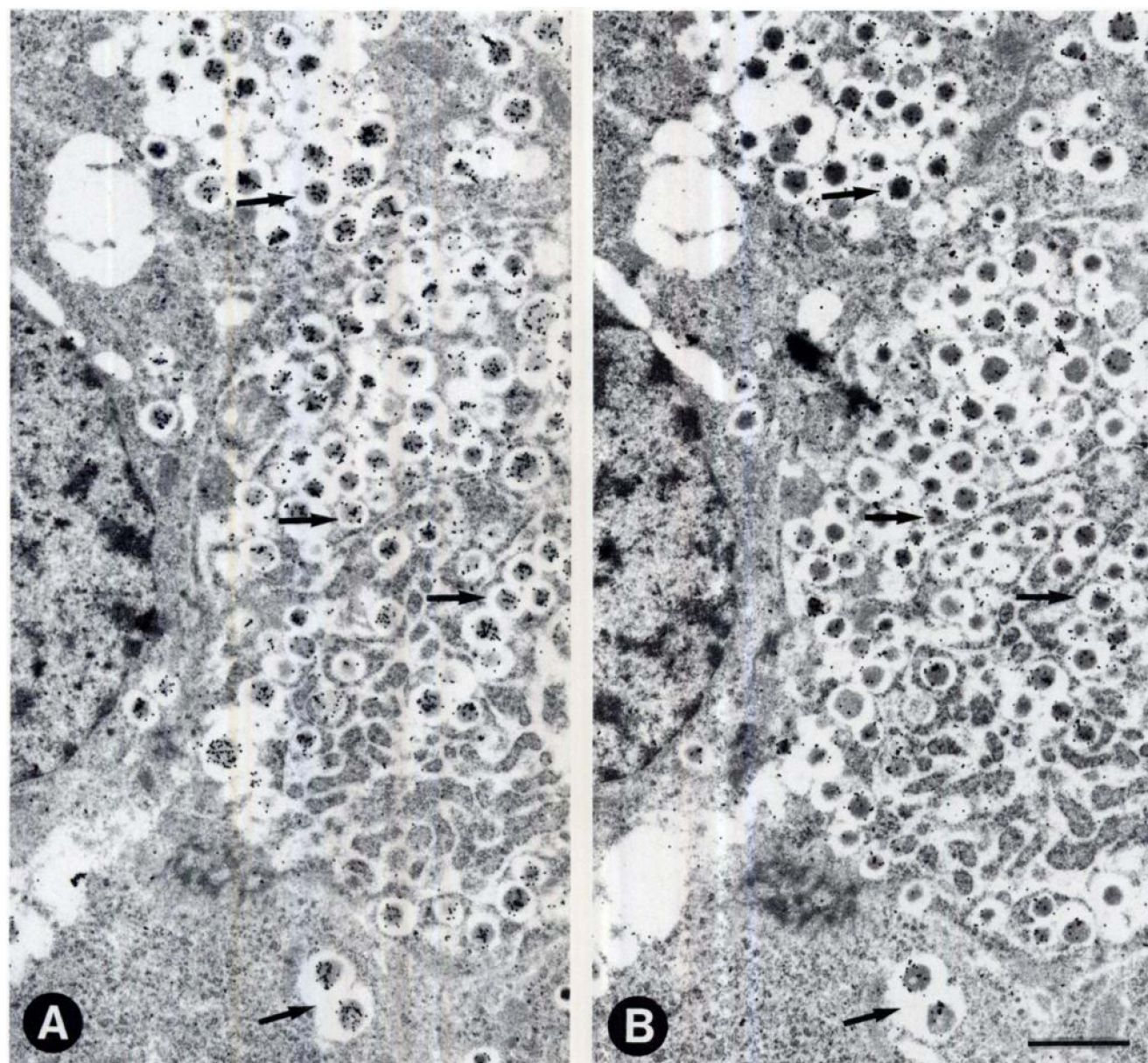


Figure 6. Photomicrographs of ultra-thin serial sections immunostained for insulin (A) and chromogranin A (B) by the protein A-gold technique. All insulin-containing vesicles also display CGA immunostains (arrows in A and B). Bar = 1 μ m.

in rat adrenal medulla chromogranin B, but not chromogranin A, makes up the major part of soluble protein in the chromaffin vesicles.

At the subcellular level, we found that CGA is stored together with the hormones within secretory vesicles. Interestingly, in the vesicle matrix the distribution of CGA differs from that of glucagon or insulin. Whereas the hormone immunoreactivity was concentrated in the dense core (insulin) or the center (glucagon) of the vesicles, CGA immunoreactivity is arranged in a characteristic way in the submembrane space because it occurs predominantly in the periphery of these vesicles.

The physiological role of CGA in endocrine cells is as yet unclear. It has recently been found that CGA acts as Ca^{2+} -binding substance within the chromaffin vesicles of adrenal medulla. In adrenal medulla, more than 99% of the intravesicular Ca^{2+} is bound to CGA and/or ATP (Reiffen and Gratzl, 1986a, 1986b; Bulenda and Gratzl, 1985). Also, the secretory vesicles of the endocrine pancreas contain large amounts of Ca^{2+} (Herman et al., 1973) but only small amounts of ATP (Leitner et al., 1975). CGA in these vesicles may therefore be the only significant Ca^{2+} -binding substance.

The function of CGA as a Ca^{2+} -binding protein may account

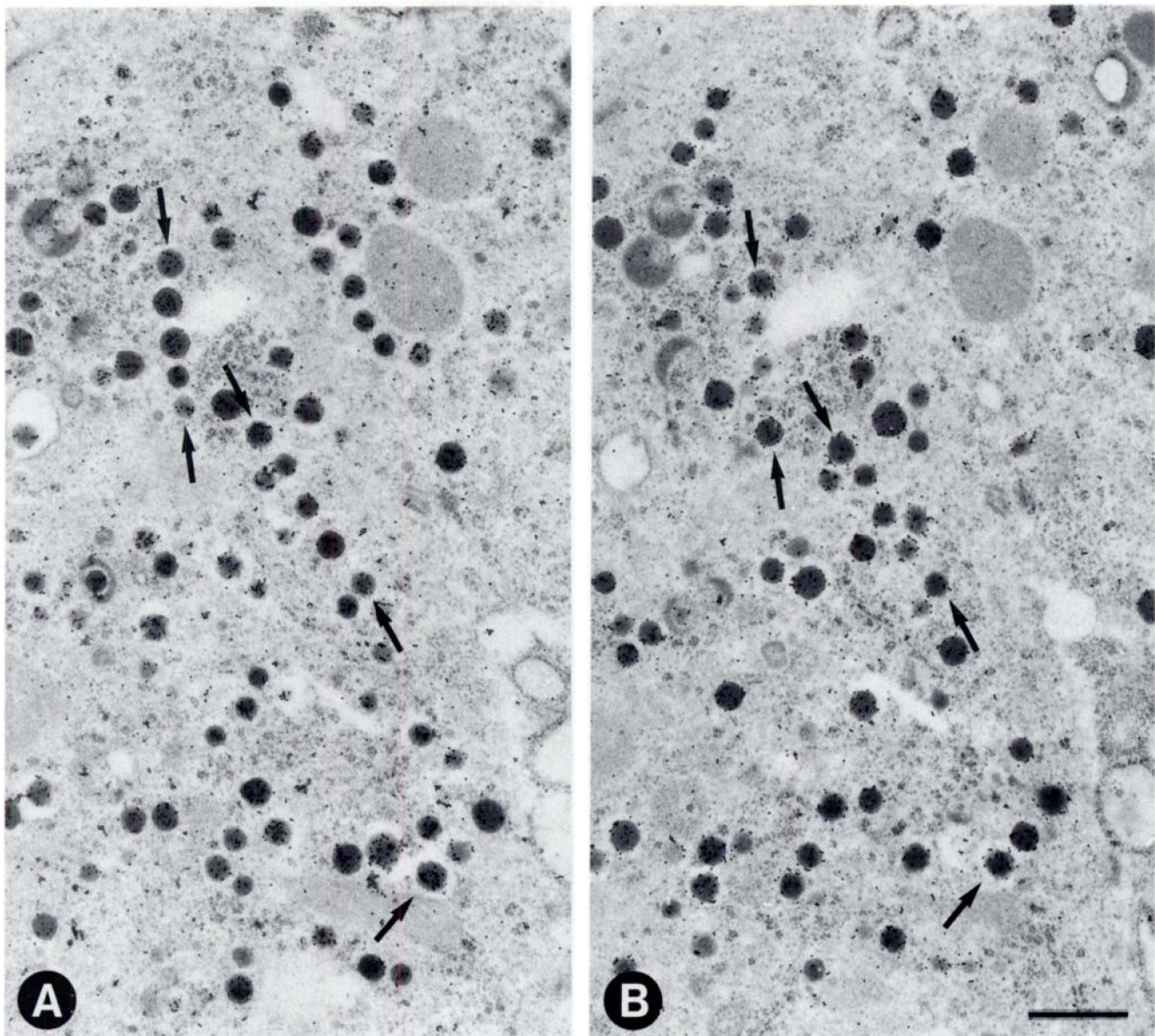


Figure 7. Photomicrographs of ultra-thin serial sections immunostained for glucagon (A) and chromogranin A (B) by the protein A-gold technique. All glucagon-containing vesicles also display CGA immunostains (arrows in A and B). Bar = 1 μ m.

for its wide distribution in the body. In addition to its presence in endocrine cells, CGA has been detected in neurons throughout the nervous system (Smogyi et al., 1984). This distribution resembles that of some membrane-bound proteins (Buckley and Kelly, 1985; Jahn et al., 1985; Wiedenmann and Franke, 1985; Matthew et al., 1981) and that of neuron-specific enolase, a cytoplasmic protein which has been found not only in nerves but also in various endocrine cells (Schmechel et al., 1978). These findings suggest that neurons and endocrine secretory cells share the same membrane, cytoplasmic, and secretory vesicle matrix proteins. Exocrine cells, which also release their secretory products via exocytosis, lack this property. Therefore, chromogranins as well as related proteins, such as the sulfated proteins recently seen in secretory ves-

cles of the adenohypophysis (Rosa et al., 1985; Rosa and Zanini, 1983), may be specific to cells of the diffuse endocrine epithelial organs (Feyrter, 1983), later described as a diffuse neuroendocrine system (APUD cell series; see Pearse 1969, 1977) or the paraneuron family (see Fujita et al., 1982; Fujita, 1976). It is therefore possible that CGA serves as a specific marker substance for these hormone- or transmitter-producing cells and that it may help to identify cells not fully characterized as members of these systems.

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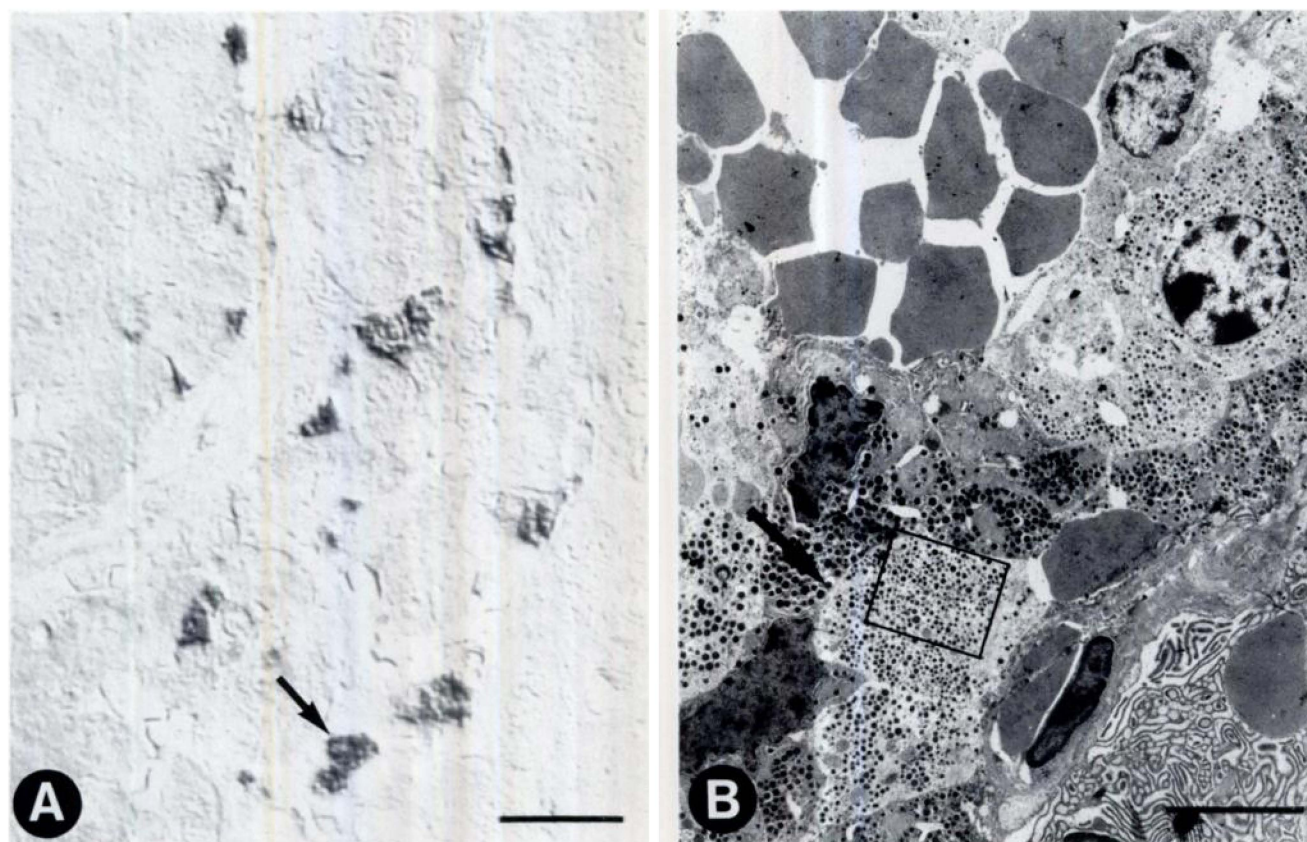


Figure 8. Photomicrographs of consecutive semi-thin and ultra-thin sections immunostained for somatostatin (A) and chromogranin A (B) by the PAP (A) and the protein A-gold technique (B). Arrows in A and B indicate the same somatostatin-containing cell. The detail surrounded in B is shown at higher magnification in Figure 9. Bars: A = 20 μ m; B = 5 μ m.

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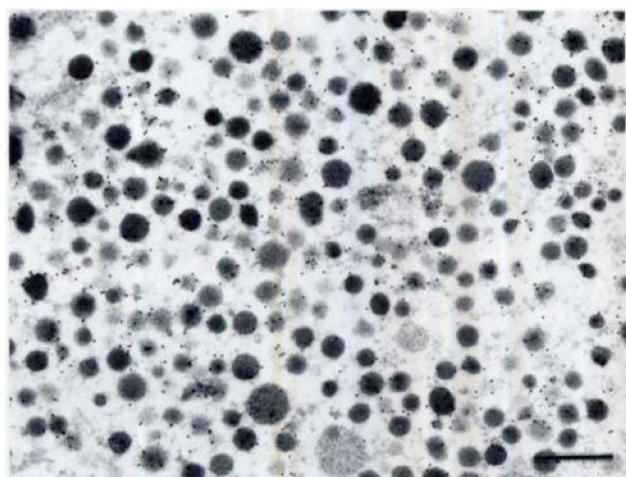


Figure 9. Enlargement of the rectangle in Figure 8B. Within the somatostatin-containing cell, the secretory vesicles also display CGA. Bar = 0.5 μ m.

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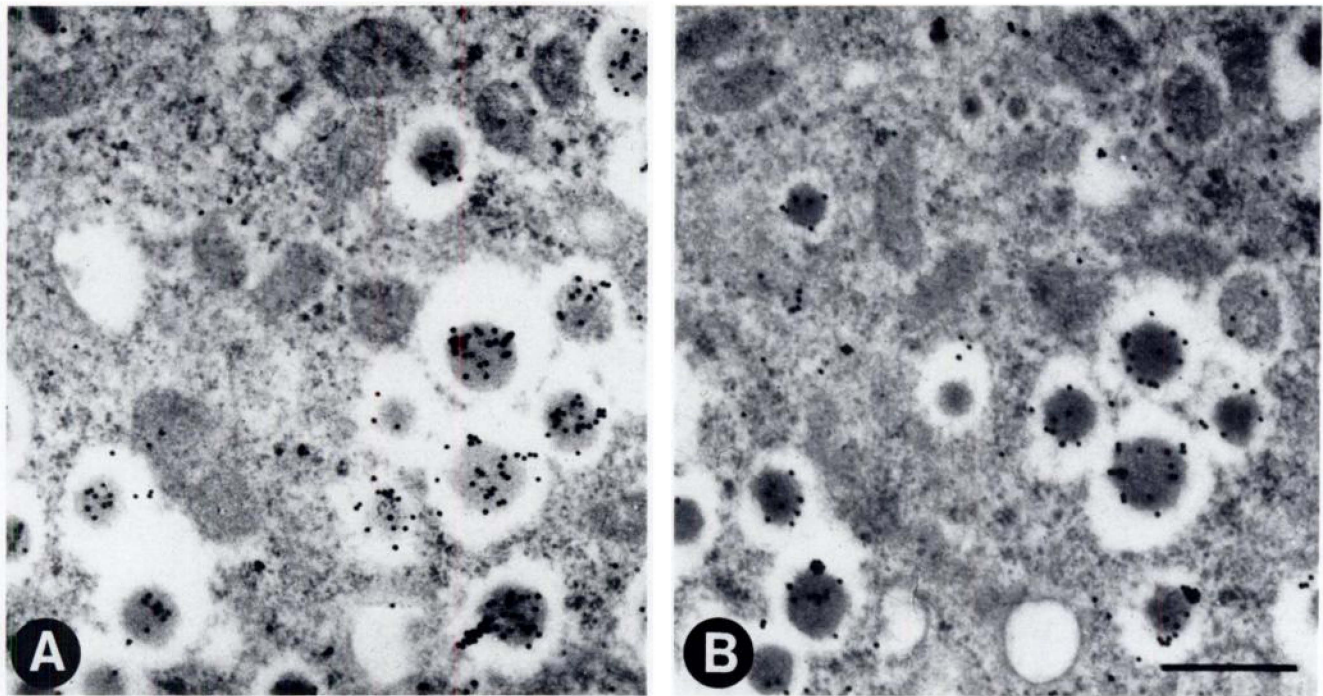


Figure 10. Photomicrographs of ultra-thin serial sections immunostained for insulin (A) and chromogranin A (B) by the protein A-gold technique. Insulin immunostains are concentrated in the core of the vesicles, while CGA-like immunoreactivity is located predominantly on the clear halo and the outer layer of the core. Bar = 0.5 μ m.

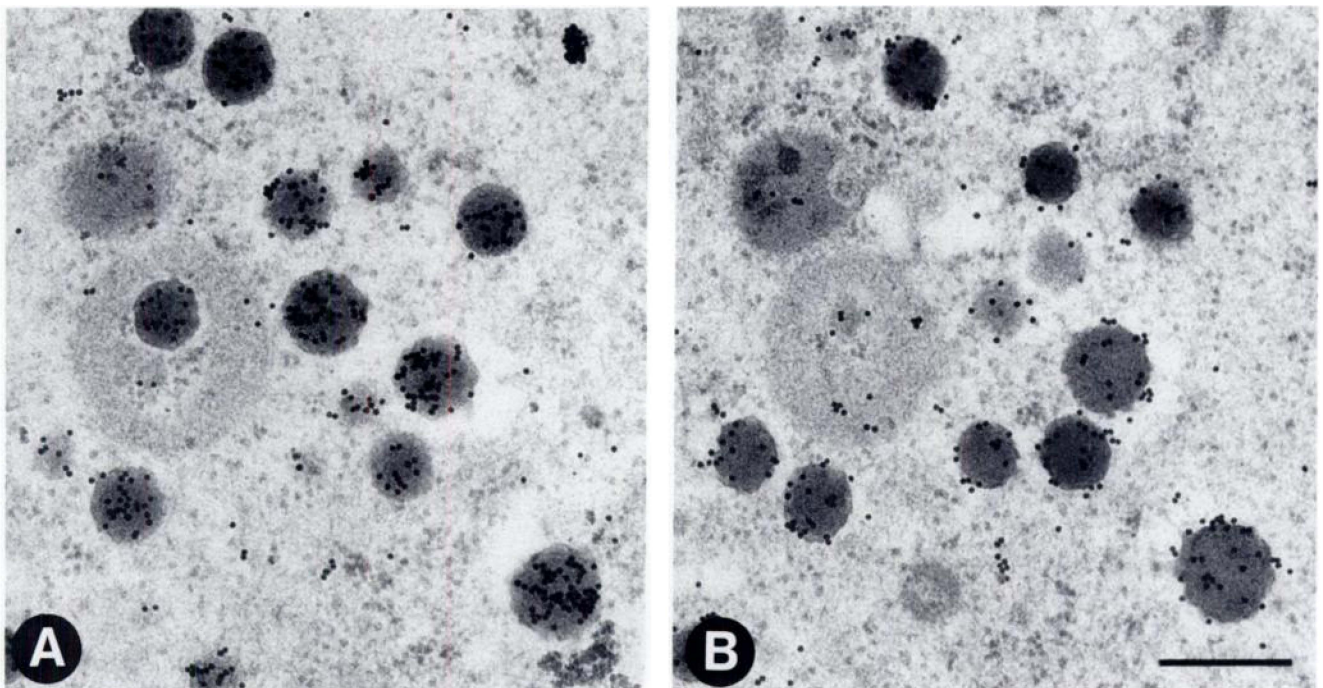


Figure 11. Photomicrographs of ultrathin serial sections immunostained for glucagon (A) and chromogranin A (B) by the protein A-gold technique. Glucagon immunostains are concentrated in the center of the vesicles, while chromogranin A-like immunoreactivity is located predominantly in the periphery (the outer 20% of the vesicle's diameter). Bar = 0.5 μ m.

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